

ADAPTATION AND IMMUNITY OF THE
LOWER ORGANISMS TO ETHYL
ALCOHOL

DISSERTATION SUBMITTED TO THE BOARD OF
UNIVERSITY STUDIES OF THE JOHNS HOPKINS
UNIVERSITY IN CONFORMITY WITH THE
REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

BY
J. FRANK DANIEL

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BIOGRAPHICAL NOTE

J. Frank Daniel was born at Ofallon, Missouri, July 31, 1873. His preparatory work was done in the Southern Illinois State Normal School at Carbondale, Illinois. After graduation from that Institution in 1901 he was sent by the Government to the Philippine Islands. There he remained four years, being successively teacher, principal of the Provincial High School, and acting superintendent for the Island of Cebu. He was graduated from the University of Chicago in 1906 with the degree of S.B. In 1907 he entered Johns Hopkins University. In this University he was University Fellow for the year 1907-1908 and Adam T. Bruce Fellow for 1908-1909.

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ADAPTATION AND IMMUNITY OF LOWER ORGANISMS TO ETHYL ALCOHOL

BY

J. FRANK DANIEL

A phenomenon of surpassing biological interest is the adjustment of living substance to its surroundings. That living things should so act as to promote their own welfare is indeed wonderful; that such acts are common is evident on every hand.

The darkened plant creeping toward the needed light, the arctic hare at the approach of winter changing its coat of gray for one of white, living tissue in battle against arsenic or alcohol or toxins securing life against an amount perchance many times in excess of the once fatal dose—these are phenomena in adjustment common to observation, yet of the utmost significance to the well-being of the organism.

Within recent years the study of these adjustments or adaptations has received unusual attention. The subject of immunity alone, developed especially by the schools of Ehrlich and Metchnikoff has within the past two decades almost attained the proportions of a science within itself.

Research in adaptation and immunity has been confined largely to the higher forms of life. These because of their complexity, offer many difficulties. A study even of an individual group of cells in these, as for example the red blood corpuscles, has to take into account a most complex medium—the blood stream. The protozoa on the other hand are comparatively simple and live in a medium which is as a rule readily subject to control. Because of these facts and for the added reason that a knowledge of the

single cell is fundamental, the following series of experiments have to do with the adjustment and immunization of different types of lower organisms to various kinds of substances.

The fact that the same species of protozoa may be found under widely differing environmental conditions—in fresh water or in alkaline lakes—suggests at once that what protoplasm is in kind depends much upon the experiences through which it has passed. If this be true the toleration of different environmental conditions just mentioned would probably be due to the gradual adjustment of the organisms to slight but constant changes in the surroundings. By such a process different members of a single race might finally come to live and prosper as separate types under conditions so widely different as to be mutually destructive.

That such extremes of adjustment are possible may be demonstrated in the laboratory.

Various investigators by subjecting organisms for a time to heat or cold have rendered them immune to great extremes of temperature.¹ Thus Dallinger² in a continuous series of experiments lasting through a period of several years so increased the resistance of various Flagellates to heat as to raise the killing point from 23° C. to the extreme of 70°, so that the organisms lived at a temperature 47° higher than that which formerly destroyed them.

Experiment with poisonous chemicals likewise shows that substances which are extremely destructive to living protoplasm may be rendered less injurious if the animal is subjected gradually to increasing concentrations. Davenport and Neal³ have shown that the unicellular organism *Stentor coerules* after being kept for a few days in a weak concentration of corrosive sublimate, is able to survive a stronger solution several times as long as can a control animal of the same series.

A beautiful case of adaptation has been shown by Hafkine.⁴ In his experiments a drop of natural water containing different

¹ For a reference to the literature, see Davenport and Castle. 1895, Arch. f. Entw. mechan., II Band, 2 Heft, pp. 227-249.

² Dallinger, W. H., 1880, Jour. Royal Mic. Soc., III, pp. 1-16.

³ Davenport, C. B., and Neal, H. V., 1896, Arch. f. Entw. mechan., II Band, 4 Heft, pp. 564-583.

⁴ Hafkine, W. M., 1890, Ann. de l'institut Pasteur, iv, pp. 363-379.

species of organisms was placed on a slide; near this was put two or three times as much of an artificial infusion in which animals were living. Upon connecting the two, both the animals in the natural water and those of the infusion died with marked violence. Animals from these divergent media, however, were brought by gradual change to live with impunity under the once mutually destructive conditions.

I have never found organisms under sufficiently divergent natural conditions to obtain Hafkine's results with minimal amounts of media, but with a small quantity of a normal culture plus a larger volume of a foreign medium, I have observed similar phenomena.

My study of adaptation in lower organisms falls naturally into two divisions. Part I⁵ deals with the adjustment of *Paramecia* to distilled water. Part II, the present study, has to do with the adjustment and immunity of *Stentor* and *Spirostomum* to ethyl alcohol.

At this point I should like to express to Prof. H. S. Jennings, under whose direction the following researches were made, my highest appreciation for the sincere sympathy he has shown at all periods of my work upon this subject.

IMMUNITY OF *STENTOR* AND *SPIROSTOMUM* TO ETHYL ALCOHOL

I INTRODUCTION

The effect of alcohol upon living matter has long been a subject for observation and experimentation. Its marked influence upon man makes it a problem of the greatest practical concern to the race. Aside from this, the nature of its action, especially in the case of its use in moderation, is of the highest scientific interest.

Within the past decade notable advancement has been made in this latter direction. Probably the work of no single investigator has been farther reaching in its results than has that of Atwater.⁶ From his extensive and thorough-going investigations we have

⁵ Published 1908, *Amer. Jour. Physiol.*, xxiii, pp. 49-63.

⁶ Atwater, *Physiological Aspects of the Liquor Problem*, vol. ii, 1903.

learned much of the real nature of the relation that alcohol bears to the process of metabolism. The former notion that alcohol facilitated the storing up of fat by retarding metabolism has largely given place to the view that alcohol is itself oxidized in the body, thus preventing or retarding the oxidation of other materials. Further than the fact of its oxidation but little is known.

In the following investigations I have undertaken a study of the general effects of alcohol upon single-celled organisms. For work of this sort much depends upon the type of cell selected. Probably no single requisite is more important than that the organism be of sufficient size to permit ready determination of the moment of death. The blue Stentor (*S. coeruleus*) meets this requirement admirably. In addition, its habit of attachment gives it a marked advantage over more active infusoria, and its characteristic reaction to light makes it easily obtainable in larger numbers relatively free from débris.

In determining the fatal dose of alcohol for Stentor it will be well briefly to survey the effects of weak percentages in general.

II PRELIMINARY EXPERIMENTS

A Effects of Minimal Amounts of Alcohol

Alcohol even in very low percentages is generally regarded as a "protoplasmic poison." Its deleterious effects in small doses depend much, however, upon the kind of organism studied.

Hodge⁷ in experimenting upon developing yeast found a decrease in division, in solutions containing as low as $\frac{1}{100}$ of 1 per cent of alcohol, the average number of cells in this strength being only 992 per cubic centimeter as compared with 2061 under normal conditions. On the other hand Maltaux and Massart⁸ for *Chilomonas* and Woodruff⁹ for "one period of the life cycle" in *Paramecium* have shown that alcohol increases the division rate. In *Paramecium* it is further important to note that the increase was lost after a time.

⁷ Hodge, 1897, Pop. Sci. Monthly, vol. 1, pp. 594-603.

⁸ Maltaux, Marie et Massart, Jean. 1906, Recueil de l'Institut Botanique, vi, pp. 269-421.

⁹ Woodruff, L. L., 1908, Biol. Bull., xv, pp. 85-104.

Richardson¹⁰ in determining the death-point for a fresh-water medusa found that from $\frac{1}{80}$ per cent to $\frac{1}{10}$ per cent of alcohol proved lethal to this form within a short period of time.

Reid Hunt¹¹ in his fascinating *Studies in Experimental Alcoholism* has obtained in higher animals the first positive evidence, so far as I am aware, of direct injury due to minimal doses of alcohol. These experiments upon white mice made it evident that amounts of ethyl alcohol far too small to produce indications of intoxication are capable of rendering the animal more susceptible to a definite poison,—acetonitrile.

In my experiments I have found protozoa comparatively resistant to weak concentrations of alcohol. In solutions of 1 per cent or lower, the organisms often lived in a prosperous condition for weeks at a time. Solutions of alcohol as high as 4 per cent in strength were in all cases early destructive of the protoplasm of *Stentor* and percentages of 2 and 3 per cent produced death after a period of six and two hours, respectively.

B Reaction to Stronger Percentages

I Description

In the following study two very different strains of *Stentor*—which we may designate as E and F—were employed. These showed marked differences in their reactions to ethyl alcohol.

Type E, composed of cells of immense size, deeply pigmented, and actively free-swimming, came from a black infusion of decayed vegetable material. Type F on the other hand—composed of cells of medium size, and usually attached, grew in a clear medium of tap water and *Chara*. These latter cells maintained a sturdy condition with slow rate of division for long periods of time.

The two types in a sublethal medium (e.g., 1 per cent alcohol) showed the following characteristic differences in behavior. The large cells E upon subjection to the alcohol were stimulated to

¹⁰ Richardson, 1888 (July), *Asclepiad*.

¹¹ Hunt, Reid, 1907, Bull. No. 33, Hyg. Lab. U. S. Pub. Health and Mar. Hosp. Serv., Washington, D. C.

marked activity. After having remained in the medium for a day an acceleration in division was observed which resulted in cells of smaller size. Type F, on the contrary, in this weak solution of alcohol showed slight increased activity upon subjection, and practically no increase in the rate of division upon remaining in the medium for a short period of time.

When these two types had been kept in a weak solution for some time, and were then placed in stronger alcohol, they showed still more important differences. Type F gained a marked immunity as a result of remaining in the weak solution, while type E showed very little effect of this sort. This difference will be brought out in full in the following investigations.

2 Method of Studying Immunity

Tests for the immunity resulting from a weak solution of alcohol may be made in one of two general ways. Either (1) the comparative resistance period of alcoholized and control (normal) animals to a known fatal percentage may be determined, or (2) the ability of alcoholized animals to live in a solution which was previously destructive may be tested.

The two may be stated more comprehensively in the following questions:

1 What is the effect produced upon the general resistance of an organism by rearing it in a weak chemical, for example alcohol, and testing it to a strong solution of the same substance.

2 Can animals by living in a weak concentration of a substance be made to survive a stronger percentage of the same? For example, can an organism which is normally killed within a few hours by a 2 per cent solution of alcohol be made to live in the same strength?

3 Experimental

As has been mentioned, the answer to these questions depends upon the animals used. Type E, kept for different periods of time in a weak medium and then tested to a stronger percentage, did not give marked evidence for immunity on either of the tests mentioned above. Type F, on the other hand, reared similarly,

gave clear evidence of immunity when tested by either of the methods. This will be seen from the two experiments which follow.

The first of these, in which F was tested for an increased resistance to a fatal dose, was made in the following way:

Into each of two dishes, designated respectively as A and C, was put 5 cc. of a natural culture medium, containing twenty normal Stentors. To A was then slowly added 1 cc. of 6 per cent alcohol, forming a 1 per cent acclimatizing medium. After a few days the animals from A (acclimatized to 1 per cent alcohol) and from C (control) were tested to a 6 per cent concentration—each experiment consisting of a drop (either of A or of C, containing a single Stentor) added to 1 cc. of 6 per cent alcohol. The death-point was taken as the instant at which ciliary movement ceased.¹² The period of resistance—the number of seconds that the organism survived—was marked in seconds.

The experiment shows the following comparative results:

Experiment I.

RESISTANCE OF STENTOR OF TYPE F TO 6 PER CENT ALCOHOL AFTER LIVING 4 DAYS IN 1 PER CENT ALCOHOL

<i>A Four Days in 1 Per Cent Alcohol</i>		<i>C Control Animals</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	150	Exp. 1 cilia stop.....	100
2 cilia stop.....	130	2 cilia stop.....	145
3 cilia stop.....	300	3 cilia stop.....	120
4 cilia stop.....	720	4 cilia stop.....	120
5 cilia stop.....	220	5 cilia stop.....	275
6 cilia stop.....	300	6 cilia stop.....	225
7 cilia stop.....	410	7 cilia stop.....	150
8 cilia stop.....	160	8 cilia stop.....	115
9 cilia stop.....	350	9 cilia stop.....	225
10 cilia stop.....	270	10 cilia stop.....	150
Average resistance = 301		Average resistance = 162.5	

Thus the animals which had been kept for a brief time in a 1 per cent solution resisted 6 per cent alcohol on the average 301

¹² It will be observed from following work that the cilia in different regions of the body show different powers of resistance, depending upon the substance used. Thus, in the case of ethyl alcohol the peristomal cilia are usually active longest, while in glycerine the body cilia continue long after the peristome is lost.

seconds, while normal animals survived but 162.5 seconds—an increase of resistance for the acclimatized individuals of nearly 2 to 1.

Experiment II. Equally satisfactory evidence of immunization appeared in Stentors of this type when tested as to their ability to live in stronger solutions of alcohol. In this experiment the following method was used:

Stentors of type F were kept for more than three weeks in a 0.5 per cent solution of alcohol. These were then put into 1 per cent for three days. From this they were transferred after two days to a 2 per cent concentration. We have already seen (p. 575) that 2 per cent alcohol kills unacclimatized Stentors of this type within six hours. But those transferred from the 1 per cent solution were in good condition at the end of the following day. At the end of the second day the animals were still in normal condition. At this time fourteen of them were transferred a third time, to a solution 3 per cent in strength. On the following morning twelve of these were dead; two, however, were alive and swimming at a rapid rate. These survived the greater part of the day.

Thus both methods of testing show that an increased resistance was produced by remaining in the weak alcoholic solution. It is evident that this caused some marked regulatory change in the animals by which they were better able to resist the ill effects of the stronger percentages of alcohol.

Following the same general plan we may now make a closer study of both types E and F.

III DETAILED EXPERIMENTS

A Method and Technique

Following the preliminary experiments, a second series was made in which the greatest care was taken to control the possible sources of error, such as evaporation and dilution of the killing fluid, fluctuations in temperature and variations in the organisms themselves.

In order to prevent error due to a weakening of the alcohol only freshly made solutions were employed. A coverslip which could be sealed in case of prolonged experimentation was used throughout the series.

Upon adding the organisms to the killing fluid considerable dilution is possible. To avoid weakening of the killing fluid either by the addition of more of the culture medium or less of the test fluid a definite amount of culture medium—a capillary drop—was added to a fixed quantity—one-fourth cubic centimeter—of the killing fluid.

Another source of error, more difficult to control, derives from the condition of the organisms themselves. A study of different strains of *Paramecia*¹³ has shown that animals adjusted to different media may react very differently to a given stimulus. Only animals of the same strain, therefore, should be used when a comparative study of the effects of different concentration is desired.

It is further of importance that a careful selection of typical animals, even from the same strain, be made. In these experiments the greatest care has been exercised to reject as abnormal all organisms showing division, regeneration, marked variations in color, considerable differences in size¹⁴ and the like.

The striking differences in reaction seen in animals tested at different degrees of temperature make the control of variable temperatures of the greatest importance.

In a room rapidly heated and ranging from 20° to 22° C. I have often found the culture media and reagents as low as 16° or 17° C. In a condition of this kind the organisms will during the experiments be tested at temperatures varying four or five degrees. With extremes so great as these marked fluctuations in resistance follow which make imperative the discarding of "room temperature" as manifestly too indefinite to be of service in work of this kind. In the following series all materials—reagents, culture media and the like—were kept at a constant temperature of 23° C.

¹³ In part i. *Loc. cit.*

¹⁴ It should be remarked that small cells are not necessarily weaker or less resistant than larger ones; the opposite is often the case. In this selection the condition sought was uniformity of size.

I wish here to express my sincerest appreciation to Professor Morse, of the Johns Hopkins Chemical Laboratory, for his helpfulness in planning a constant temperature apparatus highly satisfactory for this purpose.

B Experimental

With the various sources of error controlled in the way just set forth, the two types E and F may now be studied in detail.

I Study of Type E

In my attempts at acclimatizing Stentors of type E to alcohol various plans have been resorted to. These will be described in their proper connections.

Both methods by which type F was readily immunized to ethyl alcohol were tried for type E. The method of testing the ability of alcoholized animals to live in a stronger percentage of alcohol gave slight results for type E. Although this type was transferred with great care it was difficult to get it to live in a stronger medium than 1.5 per cent in strength. The other method in which the resistance period of the normal and acclimatized animals was compared, was adopted as the more satisfactory of the two. To test for immunity by this latter method the animals of type E were allowed to remain for short periods of time in a 1 per cent solution made by adding 6 per cent ethyl alcohol to their normal culture medium.¹⁵ The organisms were then tested as to their resistance to a fatal dose.

The results of the tests are shown in the experiments which follow:

¹⁵ Different percentages were tried for making up the acclimatizing medium and 6 per cent was adopted as less likely to produce injury when first added.

Experiment III

RESISTANCE OF STENTORS OF TYPE E TO 6 PER CENT ALCOHOL AFTER LIVING 3 DAYS IN 1 PER CENT ALCOHOL

<i>A Three Days in 1 Per Cent Alcohol</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	225	Exp. 1 cilia stop.....	235
2 cilia stop.....	250	2 cilia stop.....	250
3 cilia stop.....	225	3 cilia stop.....	300
4 cilia stop.....	390	4 cilia stop.....	320
5 cilia stop.....	435	5 cilia stop.....	405
6 cilia stop.....	480	6 cilia stop.....	330
7 cilia stop.....	535	7 cilia stop.....	360
8 cilia stop.....	285	8 cilia stop.....	240
9 cilia stop.....	595	9 cilia stop.....	140
10 cilia stop.....	330	10 cilia stop.....	240
Average resistance = 375		Average resistance = 282	

From this experiment at least two things are evident. First, the resistance of normal animals of this strain is unusually high as compared with that of type F, and secondly, the evidence for immunity is far less marked.

These animals, however, had remained in the acclimatizing medium only three days. Possibly a longer period is needed for the acclimatization of so strong a strain.

The same series at the end of the fifth day gave the following result:

Experiment IV

RESISTANCE OF STENTORS OF TYPE E TO 6 PER CENT ALCOHOL AFTER LIVING 5 DAYS IN 1 PER CENT ALCOHOL

<i>A Five Days in 1 Per Cent Alcohol</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	255	Exp. 1 cilia stop.....	255
2 cilia stop.....	395	2 cilia stop.....	240
3 cilia stop.....	520	3 cilia stop.....	360
4 cilia stop.....	230	4 cilia stop.....	380
5 cilia stop.....	315	5 cilia stop.....	150
6 cilia stop.....	380	6 cilia stop.....	330
7 cilia stop.....	410	7 cilia stop.....	205
8 cilia stop.....	210	8 cilia stop.....	405
9 cilia stop.....	490	9 cilia stop.....	260
10 cilia stop.....	300	10 cilia stop.....	330
Average resistance = 350.5		Average resistance = 291.5	

Instead of giving clearer evidence, this shows the maximum resistance found for control animals in the entire study, and an increase for the acclimatized animals which is lower than in the first experiment.

Whether a high normal resistance in any way obscures the degree of immunity actually present is a further question which may be tested by the use of a stronger killing fluid—thus reducing the period of resistance.

Animals kept in a 1 per cent acclimatizing medium for four days, then tested to an 8 per cent killing solution should be satisfactory for a determination of this point. Such a condition follows:

Experiment V

RESISTANCE OF STENTORS OF TYPE E TO 8 PER CENT ALCOHOL AFTER LIVING 4 DAYS IN 1 PER CENT ALCOHOL

<i>A Four Days in 1 Per Cent Alcohol</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	120	Exp. 1 cilia stop.....	125
2 cilia stop.....	30	2 cilia stop.....	30
3 cilia stop.....	30	3 cilia stop.....	20
4 cilia stop.....	25	4 cilia stop.....	35
5 cilia stop.....	30	5 cilia stop.....	20
6 cilia stop.....	30	6 cilia stop.....	30
7 cilia stop.....	40	7 cilia stop.....	60
8 cilia stop.....	20	8 cilia stop.....	35
9 cilia stop.....	105	9 cilia stop.....	60
10 cilia stop.....	35	10 cilia stop.....	30
Average resistance = 46.5		Average resistance = 44.5	

The results as given in the foregoing experiment, however, show that no advantage in this case followed shortening the period of resistance. On the other hand, the experiment gives much less evidence for immunity than either the first or the second.

After trying many cases all terminating as did Experiments III to V in marked variability from slight to doubtful acclimatization, it was thought that the low degree of increase might be due to injury resulting from rapid subjection to a 1 per cent acclimatizing medium. To obviate this, a method was adopted by which small and increasing amounts of alcohol were added so gradually to the normal media as to insure no injury from a too rapid sub-

jection. This method consisted in the addition on succeeding days of 4, 6 and 10 cc. of a 6 per cent concentration to 100 cc. of a normal culture medium.

Animals thus carefully reared on a gradually increasing scale for the same number of days as Experiment III gave the following results:

Experiment VI

RESISTANCE OF STENTORS OF TYPE E TO 6 PER CENT ALCOHOL AFTER 3 DAYS IN AN ACCLIMATIZING MEDIUM GRADUALLY BROUGHT UP TO 1 PER CENT

<i>A Three Days in Weak Alcohol</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	265	Exp. 1 cilia stop.....	230
2 cilia stop.....	170	2 cilia stop.....	240
3 cilia stop.....	305	3 cilia stop.....	230
4 cilia stop.....	340	4 cilia stop.....	250
5 cilia stop.....	255	5 cilia stop.....	355
6 cilia stop.....	240	6 cilia stop.....	340
7 cilia stop.....	90	7 cilia stop.....	360
8 cilia stop.....	160	8 cilia stop.....	75
9 cilia stop.....	330	9 cilia stop.....	245
10 cilia stop.....	420	10 cilia stop.....	255
Average resistance = 257.5		Average resistance = 258	

From the above it is reasonably certain that the low increase in resistance shown in Experiment III was not due to an injury attributable to the 1 per cent acclimatizing fluid, for in the same strength, to which the animals were subjected so gradually as to be without injury, no acclimatization whatsoever resulted.

Since no injury and very doubtful acclimatization is evident in a medium of 1 per cent strength, the question naturally follows: May not the low degree of immunity be due to the fact that the medium is too weak to produce an immunizing effect?

To test this, Stentors were brought through a graduated series of transfers to the highest percentage in which they could live without apparent injury—a 1.5 per cent solution. A test to 8 per cent alcohol of animals from this medium resulted as follows:

Experiment VII

RESISTANCE OF STENTORS OF TYPE E TO 8 PER CENT ALCOHOL, AFTER LIVING 4 DAYS IN 1.5 PER CENT ALCOHOL

<i>A Four Days in 1.5 Per Cent.</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	40	Exp. 1 cilia stop.....	50
2 cilia stop.....	90	2 cilia stop.....	35
3 cilia stop.....	45	3 cilia stop.....	75
4 cilia stop.....	75	4 cilia stop.....	55
5 cilia stop.....	30	5 cilia stop.....	30
6 cilia stop.....	35	6 cilia stop.....	105
7 cilia stop.....	45	7 cilia stop.....	130
8 cilia stop.....	35	8 cilia stop.....	115
9 cilia stop.....	50	9 cilia stop.....	35
10 cilia stop.....	35	10 cilia stop.....	25
Average resistance = 48.		Average resistance = 65.5	

In Experiment VII, although the animals had been kept in the strongest medium that they could withstand, still no immunity was shown. On the contrary, the alcoholized animals were actually less resistant than were those of the control.

Briefly summarizing the results of the five experiments (III to VII) it is to be noted that only III and IV gave evidence of immunity; that V, although positive in resistance, was so low as to fall easily within the experimental error; while VI showed no increase whatsoever, and VII gave a higher resistance in its control than in its alcoholized animals.

It will be observed that the foregoing series of experiments on type E were made from the third to the fifth days of acclimatization. The same general results are obtained, however, if tested either earlier or later.

A final study of this type may be added in which tests were made after the second, fifth and seventh days of acclimatization. These animals were reared in a fluid gradually brought up to 1 per cent, and were tested to a 6 per cent solution of alcohol.

The results are briefly shown in seconds of resistance in the following study made on the different days:

Experiment VIII

RESISTANCE OF STENTORS OF TYPE E TO 6 PER CENT ALCOHOL AFTER LIVING IN 1 PER CENT ALCOHOL. A = ANIMALS THAT HAVE LIVED IN ALCOHOL. C = CONTROL ANIMALS

<i>Second Day</i>		<i>Fifth Day</i>		<i>Seventh Day</i>	
A	C	A	C	A	C
<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>
410	175	310	260	220	210
265	135	275	280	425	270
175	215	410	190	235	355
220	290	255	350	330	125
95	360	190	460	350	380
120	115	310	280	230	135
290	375	305	110	385	250
450	120	160	355	250	390
250	330	320	150	225	220
340	425	275	220	320	250
Average resistance = 261.5		281	265.5	297	258.5

The outcome of this series adds little, if any, evidence for acclimatization. While in the three cases the animals of A, as in preceding experiments, show a slight increase in resistance, this is not sufficiently high, with a possible exception of the seventh day, to be at all convincing.

In summarizing the entire work done upon type E, it will be recalled that when reared for different periods of time either in weak or in stronger percentages of acclimatizing media and tested to different strengths of the killing fluid, this type gave considerable increase in resistance in only a few cases. A discussion of its tolerance may, therefore, be confined largely to a consideration of the first two (III and IV) and to the last experiment (VIII). The others, although often positive, fall easily within the possible experimental error, and may, as a consequence, be classed as unconvincing.

Since what is true for III is true also for Experiments IV and VIII, our study may further narrow itself down to a consideration of Experiment III alone.

The highest increase found during the entire study of E is that

given in Experiment III. This gives a ratio of 1.3297 + to 1 for the resistance period of the acclimatized animals as compared with that for the controls. This, however, as we shall see later, is much less marked than the results of even the least striking experiments for type F. But in degree it might well be classed as a case of immunity were it but constant. Ranging, however, from this maximum down were other experiments of the same kind which showed less or even no increase in resistance whatsoever. The latter extreme is shown in Experiment VI.

This variability of results was so noticeable that at no time has the evidence for the immunity of this type been convincing.

2 Study of Type F

From the preliminary study we saw that the animals of type F responded to acclimatization in a weak medium of alcohol by an increase in the resistance to a known fatal dose from a period of 162 seconds to 301 seconds. Further, when this type was kept for a longer period in a weak medium which was gradually increased in strength, it finally came to live in a concentration sufficient to have proved destructive before acclimatization.

We may now proceed to a study of type F, in which something of the nature of the immunization—when it begins, when it reaches its maximum degree of increase, and the like—may be sought.

The method employed in the preliminary experiment as modified in the study of type E, was followed in detail. Of the two tests for immunity given, that depending upon the increased period of resistance to a fatal dose was adopted as being the more advantageous, especially in point of time.

Incipient immunity may vary considerably as to the time of its appearance. In this range of variability several factors are involved.

One strain of animals at a given time may show immunity slightly advanced before another strain of the same type has ceased being stimulated to activity from subjection to the medium. What is true of different strains is equally true of individuals of the same strain. In other words single cells, like higher forms, show marked

individual differences in their susceptibility to alcohol—some quickly adjusting themselves to it, others doing this with greater difficulty.

Similar variability in results may be due to the fact that the acclimatizing medium is too strong or too weak. In either case there may be entire lack of immunity, or immunity may be deferred. Lack of immunity may be due either to a medium too weak to produce any effect, or one so strong as to produce lasting injury. Deferred immunity may be due to the fact that the medium is so weak that a long period is necessary for producing an acclimatizing effect, or it may come in a stronger solution when the first effect is injurious, but is later replaced by the acquirement of immunity.

In several cases I have noticed that animals tested soon after subjection to the acclimatizing medium showed a decrease in resistance. This in some cases lasted several hours; in others it was of short duration and was followed by evident adjustment.

In an acclimatizing fluid of medium strength, an early evidence of immunity may be expected from average animals by the end of the fourth or fifth hours.

The degree of possible tolerance reached evidently depends upon factors similar to those which we have just set forth. Among these may be especially mentioned—the effects of different percentages of the acclimatizing media, the period of time during which the animals are subjected to such media, and the condition of the organisms at the time of subjection.

In my work upon type F, a medium of 1 per cent alcohol has been found most satisfactory. It has, therefore, been adopted as the standard acclimatizing fluid in which all of the animals of A, with but few exceptions, have been reared. If the medium in which the animals are kept be of a strength lower than 1 per cent the immunity produced by it may be expected to be proportionately less in degree at a given time than that from a 1 per cent medium—provided, of course, the strain suffers no permanent injury from this latter strength.

Using then a 1 per cent solution of alcohol as a unit we may pro-

ceed to experiments which determine the relation that slightly differing strengths bear to different degrees of immunity.

In a solution of 0.5 per cent the evidence for adjustment to alcohol though sometimes slight is usually clear after a short time. In this strength evidence may be expected which by the end of the fourth day is definite. By this time the degree of immunity produced by two acclimatizing media—for example one of 0.5 per cent and the other of 1 per cent strength—should be sufficient to be compared, and their differences noted.

An experiment follows in which animals from 0.5 per cent and 1 per cent media were tested to 6 per cent killing fluid. Both were further controlled by the resistance of normal animals.

Experiment IX

RESISTANCE OF STENTORS OF TYPE F TO 6 PER CENT ALCOHOL AFTER LIVING 4 DAYS IN SOLUTIONS OF DIFFERENT STRENGTHS

A Four Days in 1 Per Cent Alcohol

	Seconds
Exp. 1 cilia stop.....	190
2 cilia stop.....	330
3 cilia stop.....	140
4 cilia stop.....	180
5 cilia stop.....	350
6 cilia stop.....	160
7 cilia stop.....	195
8 cilia stop.....	180
9 cilia stop.....	240
10 cilia stop.....	330

Average resistance = 229.5

A Four Days in 0.5 Per Cent Alcohol

	Seconds
Exp. 1 cilia stop.....	120
2 cilia stop.....	185
3 cilia stop.....	260
4 cilia stop.....	325
5 cilia stop.....	130
6 cilia stop.....	80
7 cilia stop.....	130
8 cilia stop.....	160
9 cilia stop.....	200
10 cilia stop.....	135

Average resistance = 172.5

C Control

	Seconds
Exp. 1 cilia stop.....	130
2 cilia stop.....	85
3 cilia stop.....	285
4 cilia stop.....	160
5 cilia stop.....	90
6 cilia stop.....	110
7 cilia stop.....	110
8 cilia stop.....	265
9 cilia stop.....	230
10 cilia stop.....	70

Average resistance = 153.5

Stentors of this type with a normal resistance averaging 153.5 seconds show in a 0.5 per cent solution a gain in resistance which though slight is nevertheless constant and typical. In a 1 per cent medium the resistance is increased to 229.5 seconds. Although the experiment was selected as one giving low results for the type, nevertheless it denotes for the animals of the 1 per cent solution a substantial gain over animals both of the control and of the 0.5 per cent medium.

But the point of most interest is that the two media, though differing but slightly in strength, show a corresponding difference in degree of immunity. From this it is seen that subjection to two media of different strengths for a definite time gives corresponding differences in the increase of immunity.

Time as a Factor in the Degree of Immunity Produced. We have now established two points. These are that at the end of four or five hours immunity normally begins and that at the end of the fourth day this is definite and considerable. The progress from the one to the other may now be noted.

The difference in degree of adjustment due to the same medium at the end of a few hours and again at the end of a few days, shows that immunity increases as the period in the acclimatizing fluid is lengthened. This is true, however, for only a limited time.

In an acclimatizing medium of average strength the adjustment which as we saw comes on at the end of the first few hours has by the end of the first day become sufficiently clear not to be mistaken. As an example of this and its subsequent history a test series may be given, which gave the highest immunity found for the type. In this the animals had at the end of the first day a resistance of 166 seconds. The resistance had on the third day increased to 249 seconds. On the fourth day a notable rise was shown in which the killing time for the acclimatized organisms reached a maximum of 334 seconds, the control at the same time showing a resistance of 160 seconds.

By the fourth day in this and subsequent experiments it was found that immunity had reached a high degree of constancy. For this reason, in the following experiments on this type, the fourth day has been selected as the time at which tests requiring a maximum degree of immunity were made.

The progress of immunity from the first to the fourth day, while showing various fluctuations, increases with a considerable degree of constancy. This may be shown in the following experiment, which is in general typical for others of the same series. In this the animals were reared in a 1 per cent medium and tested to 6 per cent alcohol on the first, second, third and fourth days of their acclimatization.

Experiment X

RESISTANCE OF STENTORS OF TYPE F TO 6 PER CENT ALCOHOL AFTER DIFFERENT PERIODS IN 1 PER CENT ALCOHOL. A = ACCLIMATIZED ANIMALS. C = CONTROLS

First Day		Second Day		Third Day		Fourth Day			
A	C	A	C	A	C	A	C		
Seconds	Seconds	Seconds	Seconds	Seconds	Seconds	Seconds	Seconds		
220	155	150	65	210	225	330	275		
170	80	420	120	215	90	370	80		
145	140	75	100	435	75	210	190		
170	120	375	145	175	155	490	115		
125	120	205	150	210	140	270	140		
150	155	215	165	380	195	225	100		
135	130	290	125	410	195	150	120		
110	105	110	205	190	165	410	130		
235	180	100	70	330	175	300	135		
180	80	300	180	190	240	240	145		
Average resistance =		164	126.5	224	132.5	274.5	165.5	299.5	143

The two extremes—the first and fourth days—are in this experiment especially typical. On the third day—as was seldom the case—almost as high a degree of immunity is represented as on the fourth. On this day, as well as on the fourth day of the test example, the control also showed a considerable increase. In both of these cases, however, the degree of increase for the control does not approximate that attained by the acclimatized animals.

It is thus seen that the same animals kept in a given percentage of acclimatizing medium give different degrees of immunity at different periods of time.

The variability which was seen to be a prominent feature in beginning immunity is nowhere seen more strikingly than in a study of the maximum degree of immunity.

Type F illustrates this in a clear way. Animals of the same strain kept under similar external conditions and for a like period of time, varied at different times in their reactions to the same concentration. Thus type F, kept in a 1 per cent medium for four days, gave at different times average resistance periods to 6 per cent alcohol of 229.5, 301, 299.5 and 334 seconds—a difference of more than 100 seconds between the two extremes, though their controls, 153.5 and 160, were practically equal at the different times.

But not alone did type F show varying degrees of resistance at different times when tested to the 6 per cent alcohol, but it manifested to a more remarkable degree the same variability when tested to a stronger concentration. Type E as we may recall, while it showed unusually high resistance to a 6 per cent killing fluid, showed low resistance when tested to 8 per cent. Type F, on the contrary, though low to 6 per cent alcohol, sometimes gave a resistance to 8 per cent which was but little short of that given to a 6 per cent solution.

This may be shown in the following experiment:

Experiment XIa

RESISTANCE OF STENTORS OF TYPE F TO 8 PER CENT ALCOHOL, AFTER LIVING 4 DAYS IN 1 PER CENT ALCOHOL

<i>A Four Days in 1 Per Cent</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	315	Exp. 1 cilia stop.....	135
2 cilia stop.....	190	2 cilia stop.....	150
3 cilia stop.....	380	3 cilia stop.....	85
4 cilia stop.....	215	4 cilia stop.....	160
5 cilia stop.....	160	5 cilia stop.....	100
6 cilia stop.....	155	6 cilia stop.....	135
7 cilia stop.....	120	7 cilia stop.....	30
8 cilia stop.....	210	8 cilia stop.....	135
9 cilia stop.....	210	9 cilia stop.....	95
10 cilia stop.....	140	10 cilia stop.....	90
	209.5		111.5

Finding the normal resistance so high, I was interested to see whether acclimatization to a 0.5 per cent solution would show,

when compared with the effects of a 1 per cent solution, differences similar to those shown in Experiment IX (p. 588).

To this end some of the same type which had been kept in a 0.5 per cent medium were tested at the same time, with the same control.

This experiment resulted as follows:

Experiment XIb

RESISTANCE OF STENTORS OF TYPE F TO 8 PER CENT ALCOHOL, AFTER LIVING 4 DAYS IN 0.1 PER CENT ALCOHOL

<i>A Four Days in 0.5 Per Cent</i>		<i>C Control</i>
	<i>Seconds</i>	
Exp. 1 cilia stop.....	90	
2 cilia stop.....	120	
3 cilia stop.....	140	
4 cilia stop.....	105	
5 cilia stop.....	180	
6 cilia stop.....	100	C..... = 111.5
7 cilia stop.....	140	
8 cilia stop.....	160	
9 cilia stop.....	140	
10 cilia stop.....	100	
Average resistance = 127.5		

While the increase is only slightly higher than that of the control yet this is much more constant as will be seen in the slight range of variability (90 to 180 seconds). This constancy shows that in this case also different concentrations produced different degrees of immunity.

3 General Discussion of Types E and F

The degree of increase in resistance due to remaining in weak alcohol is as we have seen, very different in the two types E and F. Type E in no place indicated more than a slight and occasional evidence of immunity to alcohol. Type F on the contrary gave an increase of resistance which was considerable and constant. A low average period of resistance in type F was 229.5 seconds, while that of the control was 153.5 seconds (a ratio of 1.4951 to 1). If the same average increase of resistance were

produced in E, the latter should show in such a case as that given in Experiment III, p. 581 (the highest ever observed for E), a resistance of 421.6 seconds (control 282×1.4951) instead of 375, as actually appeared. Thus the immunity indicated was at best of a low degree. This low degree taken in connection with the inconstancy of the results for that type makes the evidence for its immunization scanty indeed. In type F, on the other hand, the increase in resistance was marked and constant, so that the immunization due to a residence in weak alcohol is evident.

Evidence as to the existence of immunity to alcohol shows itself in various other ways, besides an increase in the period of resistance. The two types E and F showed also many differences in these other respects. Thus in type E the normal resistance of the control specimens was remarkably high and constant. The average period of resistance to 6 per cent alcohol of 100 experiments with unacclimatized specimens of type E was 267.9 seconds. If this be compared with the resistance period for the controls of E, as given in the foregoing experiments, the similarity is very striking. On the other hand, the increase of resistance in type E, due to keeping the animals in weak alcohol, was very inconstant.

In these respects the animals of type F offer a contrast to those of type E. In type F the natural resistance of the control animals varied considerably at different times, but the increase in resistance due to remaining in weak alcohol was uniform, so that there was an almost constant ratio between the resistance periods of the unacclimatized and the acclimatized specimens.

Furthermore, the response of the acclimatized animals of type F to the killing fluid was different from that of unacclimatized forms. It might be supposed that an increase in resistance would manifest itself especially in making the animals better able to preserve themselves intact from the action of the drug. Such was not my observation. On the contrary the acclimatized animals of type F usually suffered an early distortion. In these animals life, to outward appearances, was well nigh extinct at the end of a few seconds—the cilia beating unsteadily. Near the end of the first minute, however, they revived and death was delayed often for a considerable period of time.

This behavior may be shown in no better way than by a detailed description of what took place in a single individual. This cell is given in preliminary Experiment 1 (individual 4), and is entirely typical of the general proceedings, excepting that its resistance period was unusually long.

Upon first subjection to the killing fluid the body of this cell remained motionless; at the end of 10 seconds a strong antero-lateral bulging occurred in the aboral region; the cilia continued to beat up to 45 seconds though their action was slow and uncertain. After surviving the shock incident to subjection to the fluid, a gradual increase in strength of stroke of the cilia followed, which finally caused the whole mass of protoplasm to vibrate in wave-like rhythm, until within a few seconds of death (at 720 seconds).

We may then say that the effects of strong alcohol upon acclimatized animals of type F were different from those upon type E in at least three ways: (1) In showing a higher degree of increase, (2) in producing greater constancy of action, and (3) in manifesting a definite method of reaction in the protoplasm.

With these considerations we may now pass to a study of another cell with the single remark that never during the months that I have worked upon type E, have I been entirely able to assure myself of its adjustment to alcohol, and at no time have I had reason to doubt such adjustment in type F.

4 A Study of Spirostomum

A further study of acclimatization to alcohol was made upon one of the largest of the single celled organisms, *Spirostomum* (*S. ambiguum*).

While this form is not found attached as in the case of *Stentor*, the disadvantage due to its activity is fully compensated for by the most definite of death-points. Subjected to a chemical stimulus *Spirostomum* begins backing, as is characteristic of many of the ciliates. If the chemical into which the organism is introduced be of sufficient strength to cause death the first sign of injury appears as a rupture at the extreme anterior end. Disintegration, beginning at this point, travels rapidly towards the posterior end

(following the direction the organism is moving) until it has consumed the entire body—leaving in its path only a granular mass of disintegrated protoplasm. Death is that unmistakable point at which the last trace of form is destroyed and at which the last cilium can be clearly seen to stop moving.

The fact that this organism can be obtained in large numbers and kept for long periods of time, together with the fact that it gives so definite a death-point, makes it in some respects preferable even to *Stentor* for work of this sort.

The *Spirostoma* of the following studies grew in a medium in which the decayed vegetable material had settled to the bottom, leaving the organisms in a relatively clear liquid. In this they flourished in great abundance, furnishing most satisfactory material for comparative studies for months at a time.

Individual variation in resistance to chemicals is in the case of *Spirostomum* most marked. Animals from the same culture, when tested to a low fatal dose, may be divided roughly into two groups—one dying within two to three minutes, the other (a smaller group) surviving in the same fluid a much longer time.

In work upon this form the larger group above mentioned has been taken as the more representative and organisms with a resistance exceeding seven minutes have not been counted. This would seem likely to produce error, for if animals with a normal resistance approaching seven minutes be raised by acclimatization to or above this maximum they would be rejected as abnormal. Those of the control, on the other hand, would not thus increase above this maximum and would therefore be counted. The elimination of the one and the counting of the other would result in a ratio between the two that would indicate less immunity than was actually present.

As a matter of fact error of this sort has been largely controlled in the following work. This was done by the use of a stronger killing fluid, which shortened the period of resistance. In an 8 per cent killing fluid it was found that only a few lived longer than seven minutes.

With these considerations we may now pass directly to the experiments.

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The method and technique developed for the study of Stentor were used as satisfactory also for a study of Spirostomum. The animals were kept in a low percentage of alcohol (1 per cent) for a short time and were then tested for their resistance to a stronger concentration. The conditions—such as the maintenance of constant temperature and the like—were similar in all respects to those of Stentor.

Experiment XII. In determining the effect of a low fatal percentage of alcohol upon Spirostomum a 6 per cent solution was first used. In this, however, it was found that, although normal animals succumbed, nine out of ten of those acclimatized were alive at the end of thirty minutes.

From this it was obvious that in order to get a quantitative statement of the resistance a stronger test fluid was needed. Thereupon an 8 per cent medium was employed with the following typical results:

Experiment XII

RESISTANCE OF SPIROSTOMA TO 8 PER CENT ALCOHOL AFTER LIVING 4 DAYS IN 1 PER CENT ALCOHOL

<i>A Four Days in 1 Per Cent Alcohol</i>			<i>C Control</i>		
		<i>Seconds</i>			<i>Seconds</i>
Exp. 1	cilia stop.....	110	Exp. 1	cilia stop.....	80
2	cilia stop.....	145	2	cilia stop.....	50
3	cilia stop.....	220	3	cilia stop.....	65
4	cilia stop.....	45	4	cilia stop.....	55
5	cilia stop.....	90	5	cilia stop.....	50
6	cilia stop.....	150	6	cilia stop.....	60
7	cilia stop.....	210	7	cilia stop.....	180
8	cilia stop.....	165	8	cilia stop.....	50
9	cilia stop.....	240	9	cilia stop.....	135
10	cilia stop.....	190	10	cilia stop.....	80
		156.5			80.5

From Experiment XII it is seen that the increase is much like that given in type F of Stentor. Although in Spirostomum there is a greater range of variability yet the striking feature in the many cases observed was that although the normal animals varied in their resistance from time to time the ratio between acclimatized and unacclimatized (control) animals remained practically the same.

From experiments in an 8 per cent solution, another point of extreme interest was observed.

Organisms often showed the first signs of injury within the usual time—one to two minutes—and disintegration followed in the usual way. Upon reaching mid-body, however, this was suddenly stopped. At the point of injury a round plug of protoplasm formed, filling up the wound. Thereupon the cilia resumed a backward stroke and the body moved forward in a normal fashion.

This phenomenon was observed again and again as the method by which the organisms often prolonged life for considerable periods of time.

In the same way that type E of Stentor was tested on the second, fifth and seventh days, we may test Spirostomum to see the general indications of resistance.

These results, shown in a condensed way in the following table, are different from those obtained in type E of Stentor.

Experiment XIII

RESISTANCE OF SPIROSTOMA TO 8 PER CENT ALCOHOL AFTER LIVING IN 1 PER CENT ALCOHOL. A = LIVING IN 1 PER CENT ALCOHOL. C = CONTROL

<i>Second Day</i>		<i>Fifth Day</i>		<i>Seventh Day</i>	
A	C	A	C	A	C
<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>	<i>Seconds</i>
45	90	195	50	210	45
205	150	240	220	260	55
110	45	75	70	195	100
55	190	70	90	330	40
90	65	215	110	55	50
200	150	255	50	90	50
120	105	70	70	190	85
95	65	255	60	60	50
225	90	205	70	190	60
95	90	70	45	105	120
Average resistance . . . 124		165	83.5	168.5	65.5

In the foregoing series only a slight increase for the acclimatized animals is shown on the second day. It will be noted, however, that the normal resistance (104 seconds) is high. In other cases

a better increase was shown at the end of the first day than is here given on the second. Probably the most typical results for the series are those on the fifth day. On the seventh a slightly higher average obtains for A, but C is less resistant. Had it been typical, however, there would still be a ratio slightly above two to one.

Another point may be noted in passing. The control animals, unlike those of Stentor, instead of increasing in resistance, showed a gradual decrease.

A further study was made in *Spirostomum* of the immunity shown at considerably later periods of time.

The greatest difficulty encountered was in keeping the acclimatizing medium A at its usual strength. In order to do this two plans were tried. In one the culture was changed every few days; in the other it was kept in ground glass vessels which were sealed and set at constant temperature until the time of experiment. The latter method was adopted as giving better results.

An experiment under these conditions follows in which the organisms were tested after eleven days in the acclimatizing and control media.

Experiment XIV

RESISTANCE OF SPIROSTOMUM TO 8 PER CENT ALCOHOL, AFTER LIVING 11 DAYS IN 1 PER CENT ALCOHOL

<i>A 11 Days in 1 Per Cent Alcohol</i>		<i>C Control</i>	
	<i>Seconds</i>		<i>Seconds</i>
Exp. 1 cilia stop.....	190	Exp. 1 cilia stop.....	30
2 cilia stop.....	50	2 cilia stop.....	70
3 cilia stop.....	85	3 cilia stop.....	25
4 cilia stop.....	95	4 cilia stop.....	160
5 cilia stop.....	95	5 cilia stop.....	120
6 cilia stop.....	390	6 cilia stop.....	30
7 cilia stop.....	45	7 cilia stop.....	65
8 cilia stop.....	285	8 cilia stop.....	60
9 cilia stop.....	150	9 cilia stop.....	50
10 cilia stop.....	70	10 cilia stop.....	30
	145.5		64

We see from this that although the resistance of the control was low, the acclimatized animals have retained their immunity for this period of time.

A later series at the end of the second week gave the following similar results:

Experiment XV

RESISTANCE OF SPIROSTOMA TO 8 PER CENT ALCOHOL AFTER 14 DAYS IN 1 PER CENT ALCOHOL

<i>A 14 Days in 1 Per Cent Alcohol</i>			<i>C Control</i>		
	<i>Seconds</i>			<i>Seconds</i>	
Exp. 1 cilia stop.....	215		Exp. 1 cilia stop.....	25	
2 cilia stop.....	60		2 cilia stop.....	125	
3 cilia stop.....	180		3 cilia stop.....	30	
4 cilia stop.....	75		4 cilia stop.....	50	
5 cilia stop.....	250		5 cilia stop.....	55	
6 cilia stop.....	230		6 cilia stop.....	50	
7 cilia stop.....	65		7 cilia stop.....	95	
8 cilia stop.....	55		8 cilia stop.....	30	
9 cilia stop.....	95		9 cilia stop.....	120	
10 cilia stop.....	50		10 cilia stop.....	30	
	127.5			61	

From these two experiments it appears probable that immunity remains as long as the killing fluid is not markedly weakened.

A study of *Spirostomum* shows a marked degree of similarity between its reaction and that of type F of *Stentor*. In both there was a well marked immunity which by the end of the fourth day had reached a degree of constancy.

The experiments just described show further that the immunity which was constant on the fourth to the seventh day was still present when tested at a much later date.

With this we may conclude the present study and pass to a consideration of another phase of the nature of immunity.

IV SPECIFICITY OF IMMUNITY

Ehrlich¹⁶ found that white mice which were immunized to *ricin* were still susceptible to another poison—that of *abrin*. The immunity conferred by the one substance in this case did not carry with it protection against a different substance. In other words its action was specific.

¹⁶ Ehrlich, P., 1891, Deutsche med. Wochenschrift no. 12 (*ricin*), no. 14 (*abrin*).

Whether the same specificity holds in the case of single cells where a lower degree of immunity obtains, is a question to which considerable interest attaches. The problem may be stated thus: Will *acclimatized* unicellular organisms, which show a stronger resistance to alcohol, also demonstrate an increased resistance when tested to a fatal dose of another and different substance?

To determine the point in question organisms which I have found could be rendered immune to ethyl alcohol have been tested: (1) to substances radically different from alcohol and (2) to substances in a way related to alcohol. In the first case normal and acclimatized animals of both *Stentor*¹⁷ and *Spirostomum* were tested to hydrochloric acid and to sodium hydroxide. In the second, they were subjected to other alcohols—methyl alcohol and glycerin.

In these studies attention has been directed to two things: First, to the physiological effects—that is, to what the organism did; and secondly, to the chemical effects, or to what changes the chemical produced.

The first point was to see whether normal and acclimatized animals acted in the same way or differently in the same killing medium. A second was to determine whether there was any difference in chemical effect upon the two sets—the acclimatized and control animals.

If such differences were noted either in behavior on the part of the organism or in action on the part of the chemical, a further proposition would be to see if any relation exists between the two.

A *The Action of Ethyl Alcohol (C_2H_5OH)*

As a standard for subsequent observation and experiment we may repeat earlier experiments in order to see what takes place when control and acclimatized animals are subjected to a fatal dose of ethyl alcohol.

Normal unacclimatized Stentors (type F) when thus subjected to a 6 per cent killing fluid showed slight or no bodily movement. At from 15 to 35 seconds there was a strong antero-lateral (aboral)

¹⁷ In this study when *Stentor* is mentioned type F is meant unless otherwise stated.

bulging and extrusion of the protoplasm, followed by a rapid loss of color. The body cilia ceased moving early, but the membranellæ continued with strong stroke until nearly the time of death.

Acclimatized Stentors, on the other hand, upon subjection to the 6 per cent alcohol usually remained motionless. Early distortion, then uncertain beat of cilia up to 45 seconds, was followed by an increase in ciliary activity. This finally became so vigorous as to shake the whole body mass of protoplasm. In this, as in our previous study, an extreme tenacity of life was manifest.

Thus both in behavior and in chemical action differences were produced. The relation the one bears to the other, however, is difficult to see.

Notwithstanding the fact that a stronger external effect was produced upon the acclimatized animals than upon the controls, the former survived a greater period of time in a lethal percentage of alcohol than did those of the control animals.

We may now examine the phenomena in acclimatized and control animals when hydrochloric acid is used as the killing fluid.

B The Action of Hydrochloric Acid (HCl)

I General Effects

Unacclimatized Stentors tested to an $\frac{M}{800}$ solution. In a concentration of this strength an early rotation was noted which ceased soon after injury began (15 to 30 seconds); the membranellæ stopped early. The body upon losing its color became brown. A little later contortions of the protoplasm were followed by a splitting away of the body mass from the cell wall and a forming of this mass into a coagulum.

After this action no ciliary motion was seen excepting in the buccal cavity.

Acclimatized Stentors. With the exception that a slightly greater activity was shown in these than in normal *Stentors* no difference either physiological or chemical could be detected.

The most striking phenomenon of the above study, observed both in normal and acclimatized animals, was the peculiar way in which ciliary activity was stopped. Just before death, pro-

nounced contortions of the protoplasm were the forerunners of a wave-like action which passed posteriorly, splitting away the endoplasm from the pellicula and forming of the endoplasm a typical coagulum. As a result of this action, all ciliary movement ceases, excepting that of the buccal pouch. (At this location, pellicula and endoplasm were not early separated.)

Destruction came about more rapidly in the case of acclimatized animals than of the controls; this may be seen from the following experiment upon resistance (Stentor). But the reason for a much earlier splitting and coagulum formation in the one case than in the other is by no means clear.

2 Effects Upon Resistance

In the following experiments upon both Stentor (type F) and Spirostomum, a notable difference was seen between normal and acclimatized animals when tested as to their endurance in a lethal dose of hydrochloric acid.

In both cases the acclimatized animals were reared in a 1 per cent medium of alcohol. These at the end of four days showed an increased resistance to 6 per cent alcohol. They were then tested to $\frac{M}{800}$ and $\frac{M}{100}$ concentrations of HCl, respectively.

Experiment XVI

RESISTANCE OF STENTOR AND SPIROSTOMUM TO HCl AFTER ACCLIMATIZATION IN A 1 PER CENT SOLUTION OF ALCOHOL

<i>a Stentor</i>			<i>b Spirostomum</i>		
Tested in $\frac{M}{800}$ Sol. HCl			Tested in $\frac{M}{100}$ Sol. HCl		
	A	C		A	C
	Acclimatized	Control		Acclimatized	Control
	to Alcohol			to Alcohol	
	Seconds	Seconds		Seconds	Seconds
Exp. 1 cilia stop.....	60	170	Exp. 1 cilia stop.....	150	150
2 cilia stop.....	210	210	2 cilia stop.....	160	160
3 cilia stop.....	180	230	3 cilia stop.....	140	140
4 cilia stop.....	50	180	4 cilia stop.....	180	180
5 cilia stop.....	40	140	5 cilia stop.....	160	235
6 cilia stop.....	40	285	6 cilia stop.....	155	150
7 cilia stop.....	40	270	7 cilia stop.....	140	160
8 cilia stop.....	50	195	8 cilia stop.....	200	180
9 cilia stop.....	70	390	9 cilia stop.....	145	260
10 cilia stop.....	45	420	10 cilia stop.....	160	170
Average resistance.... = 78.5			249	Average resistance.... = 159	
				178.5	

Both for Stentor and Spirostomum not only was there no increase in resistance of acclimated animals when tested to hydrochloric acid but there was—especially in Stentor—a clear and unmistakable weakening. Animals which normally resisted $\frac{M}{800}$ HCl for 249 seconds, if first accustomed to alcohol, showed an average endurance of only 78.5 seconds. Spirostomum, while showing less injury, in no case showed advantage by first being acclimatized to alcohol.

For Spirostomum it will be noticed that a much lower susceptibility to HCl was shown. Although its controls were tested in a medium eight times as concentrated as that used for Stentor, they gave a resistance period almost as long as did normal animals of Stentor; while its acclimatized animals were much more resistant to this solution than were similar acclimatized Stentors in the much weaker solution.

This lack of susceptibility to acid on the part of Spirostomum has in large part its explanation in the contour of the cell. In Stentor it was seen that in the formation of the coagulum the large rounded mass of endoplasm was drawn away from the pellicula, and that this separation is what causes the cessation of ciliary movement. In Spirostomum, however, the mass of endoplasm is long and columnar and forms into a coagulum more slowly and with a much less destructive effect. The coagulum in passing along the body becomes shorter and thicker. This rounding together with slight constrictions in the cell wall impedes its course, thus allowing the posterior cilia to continue beating often for long periods of time.

Thus we see that while the formation of a coagulum is destructive to Stentor, the same process gives advantages to Spirostomum when tested to a fatal percentage of hydrochloric acid.

C The Action of Sodium Hydroxide (NaOH)

I General Effects

Normal unacclimatized Stentors in a solution of $\frac{M}{100}$ NaOH gave the following characteristic reactions: Slight movement for a brief time (15 seconds) followed by a loss of the membranellæ

and a prominent protrusion of the membranellar plates. Color—a beautiful sea green—extruded. Body usually burst and rapid destruction followed—the cilia remaining active as long as a particle of form remained.

Acclimatized Stentors. Stentors which had been acclimatized in 1 per cent alcohol, upon subjection to $\frac{M}{100}$ NaOH turned rapidly around and around for a few seconds. The body wall then gave way and destruction followed with extreme rapidity.

In a comparison of acclimatized and unacclimatized Stentors two differences are noticed. Acclimatized animals show a greater activity upon subjection to sodium hydroxide and this chemical acts with greater rapidity upon the acclimatized forms.

The most notable thing observed in the study of sodium hydroxide was in relation to the pellicular membrane. If the pellicula remained intact, life was often prolonged for a remarkable period of time; if on the other hand the pellicular wall gave way, the cell was dissolved with extreme rapidity—leaving only traces of protoplasmic granules in the surrounding medium.

Rapidity of death to those animals which have remained for a brief period of time in alcohol has a possible explanation in the fact, previously noted, that acclimatized Stentors, even in alcohol, show an early distortion. A rupture of the pellicula in NaOH means immediate death. If the process of acclimatization to alcohol makes a rupture of this sort more probable, it would naturally follow that if the organisms are first subject to a weak percentage of alcohol and then tested to a fatal dose of sodium hydroxide they would meet an earlier death. This is in agreement with what is seen in Experiment XVII.

2 Effects upon Resistance

In the following experiment acclimatized and unacclimatized animals of both Stentor and Spirostomum were tested to see the period of time that they could withstand an $\frac{M}{100}$ solution of sodium hydroxide:

Experiment XVII

RESISTANCE OF STENTOR AND SPIROSTOMUM TO $\frac{M}{100}$ NaOH AFTER ACCLIMATIZATION IN 1 PER CENT ALCOHOL

<i>a Stentor</i>			<i>b Spirostomum</i>		
	A	C		A	C
	Acclimatized to	Control		Acclimatized to	Control
	1 Per Cent			1 Per Cent	
	Alcohol			Alcohol	
	Seconds	Seconds		Seconds	Seconds
Exp. 1 cilia stop.....	90	220	Exp. 1 cilia stop.....	35	30
2 cilia stop.....	180	225	2 cilia stop.....	40	30
3 cilia stop.....	135	110	3 cilia stop.....	20.	35
4 cilia stop.....	215	420	4 cilia stop.....	20	20
5 cilia stop.....	60	270	5 cilia stop.....	25	30
6 cilia stop.....	90	45	6 cilia stop.....	30	35
7 cilia stop.....	195	255	7 cilia stop.....	25	50
8 cilia stop.....	45	220	8 cilia stop.....	20	60
9 cilia stop.....	130	330	9 cilia stop.....	35	40
10 cilia stop.....	60	225	10 cilia stop.....	40	60
	—	—		—	—
Average resistance. =	126	232	Average resistance. =	29	39

It will be seen that Stentors are much less susceptible to sodium hydroxide than they were to hydrochloric acid. In a solution of NaOH eight times as concentrated ($\frac{M}{100}$) practically the same control resistance was shown as in ($\frac{M}{800}$) HCl (232 seconds, 249 seconds respectively). But that this is in no sense general for single cells may be seen from Spirostomum, which was more resistant to the acid than to an equal concentration of the base.

This similarity of susceptibility to equal concentrations of acid and base was not in Spirostomum due alone to the retarding action of the coagulum in acids, but partly also to the fact that in bases the body burst early and disintegrated with great rapidity.

In both of the foregoing studies acclimatized animals which gave an increased resistance to alcohol, when tested either to hydrochloric acid or to sodium hydroxide showed no such increase. On the contrary they invariably demonstrated a lower degree of endurance. Thus the immunizing action of the alcohol was in these cases clearly specific.

We shall now turn to a consideration of the second group of substances.

Whether animals acclimatized to weak concentrations of alcohol have gained a benefit which will help them when tested to a fatal dose of a substance kindred to alcohol is a question of closer interest. We shall first study this in one of the lower alcohols.

D The Action of Glycerin $C_3H_5(OH)_3$

I General Effects

Normal unacclimatized Stentors. Subjected to a molecular concentration of glycerin ($\frac{M}{1}$) the animals remained motionless for an instant, then suddenly began backing and contracting rapidly. Membranellæ became non-functional, usually within 45 seconds. The peristome (membranellæ) was soon lost. Signs of plasmolysis—especially in the posterior part of the body—followed. The color was retained to a marked degree. Long after the membranellæ had stopped beating the body cilia continued in activity (the opposite effect from that of ethyl alcohol).

Stentors acclimatized in 1 per cent alcohol. In the same concentration of glycerine these gave reactions which could not be distinguished from those described above.

A single peculiarity in acclimatized animals may be mentioned. In a number of cases these assumed a characteristic pipe-shaped appearance, similar to that seen when normal Stentors were kept in water of very great purity.

The most characteristic phenomenon observed from the action of glycerin was the loss of the peristome or membranellæ. This was noted alike in acclimatized and unacclimatized animals and in solutions varying in concentration from a molecular solution to a concentration of one-fourth molecular strength.

The first signs of injury to the organism came as a stoppage of these peristomal cilia. The peristome then became detached in a ribbon-like fashion and either hung from a point of attachment or, as was more usual, was entirely lost.

A phenomenon of great interest was the regeneration of the peristome after its loss in the manner just described. In a sub-lethal concentration those animals which gave off the peristome in the afternoon had by the following morning regenerated a new one.

Johnson¹⁸ has shown that the process of regeneration in *Stentor* is similar to what occurs in the intricate process of division. In the cases of loss of the peristome which I have just described, however, no active condensing and separating of the nodes of the meganucleus took place. A part of the body simply constricted off and the meganuclear nodes were seemingly undisturbed.

2 Effects upon Resistance

The animals in the following experiment were reared in 1 per cent alcohol and then tested in comparison with control specimens to a molecular solution of glycerin:

Experiment XVIII

RESISTANCE OF STENTOR AND SPIROSTOMUM TO $\frac{M}{1}$ GLYCERIN, AFTER ACCLIMATIZATION IN 1 PER CENT ALCOHOL

<i>a Stentor</i>			<i>b Spirostomum</i>		
	A	C		A	C
	Acclimatized to	Control		Acclimatized to	Control
	1 per cent			1 per cent	
	Alcohol			Alcohol	
	Seconds	Seconds		Seconds	Seconds
Exp. 1 cilia stop.....	180	240	Exp. 1 cilia stop	120	240
2 cilia stop.....	240	450	2 cilia stop.....	240	180
3 cilia stop.....	150	255	3 cilia stop.....	150	240
4 cilia stop.....	120	330	4 cilia stop.....	180	300
5 cilia stop.....	100	285	5 cilia stop.....	180	200
6 cilia stop.....	135	300	6 cilia stop.....	210	240
7 cilia stop.....	205	250	7 cilia stop.....	180	240
8 cilia stop.....	190	340	8 cilia stop.....	200	180
9 cilia stop.....	150	225	9 cilia stop.....	180	250
10 cilia stop.....	165	300	10 cilia stop.....	210	240
Average resistance. =	163.5	297.5	Average resistance. =	185	231

While, as we have before seen, no difference either in behavior or in chemical action could be detected as between unacclimatized and acclimatized animals, yet in this study on comparative resistance a difference was noticed. In a solution of the above strength both cases gave clear evidence of the specificity of the immunity

¹⁸ Johnson, Herbert P., 1893, Jour. of Morphol., viii, pp. 467-562.

due to acclimatization in 1 per cent alcohol. The resistance to $\frac{M}{1}$ glycerin was decreased by remaining in alcohol.

In a weaker solution, however, while *Spirostomum* showed a similar specificity, *Stentor* was far less regular. In an $\frac{M}{2}$ solution in one case it showed a slight advantage in its acclimatized animals ($A = 516''$, $C = 462''$). Study in a weaker solution ($\frac{M}{4}$) gave results still more difficult to interpret. Animals reared in 1 per cent alcohol gave a resistance to $\frac{M}{4}$ glycerin of 1293 seconds; those in 0.5 per cent, 1696 seconds, and those reared in normal culture medium, 1600 seconds.

Even in these cases a certain degree of specificity of immunity is evident, for in no place is there a degree of increase in the resistance to glycerin approximating that given to ethyl alcohol.

E The Action of Methyl Alcohol (CH_3OH)

I General Effects

In methyl alcohol, a lower member of the alcohol group, the following condition was found:

Normal unacclimatized Stentors subjected to an 8 per cent solution: Movement slight or animal altogether quiet; body bulging antero-laterally much as in the case of ethyl alcohol. Loss of body color; strongly marked distortion in 80 to 105 seconds; membranellæ around groove persistent; frontal field retaining color and its cilia active after the posterior part of the body was colorless.

Acclimatized Stentors. *Stentors* acclimatized in 1 per cent ethyl alcohol when subjected to 8 per cent methyl alcohol showed the following effects:

Early movement; membranellæ stopped somewhat as in glycerin; an occasional animal with membranellæ lost, but the peristome was not cast off in a ribbon-like band as occurred in glycerin.

Slight, if any, difference could be seen in the behavior of the acclimatized and the control animals in the above comparison. In both, the membranellæ had a resistance mid-way between that shown in ethyl alcohol and in glycerin.

A considerable difference was seen between the action of methyl alcohol and that of glycerin upon the color and cilia of the frontal field. Both color and ciliary activity of this region were long retained in methyl alcohol while in the glycerin both were lost at a comparatively early time.

2 Effects upon Resistance

In *Stentor* and *Spirostomum*, the comparative resistance for both normal and acclimatized animals is shown in Experiment XIX which follows. In this *Stentor* was tested to an 8 per cent concentration and *Spirostomum* to a 10 per cent solution of methyl alcohol.

Experiment XIX.

RESISTANCE OF STENTOR AND SPIROSTOMUM TO METHYL ALCOHOL, AFTER LIVING IN 1 PER CENT ETHYL ALCOHOL

<i>a Stentor</i>				<i>b Spirostomum</i>			
Tested to 8 Per Cent CH ₃ OH				Tested to 10 Per Cent CH ₃ OH			
	A	C			A	C	
	Acclimatized to 1 per cent Ethyl Alcohol		Control		Acclimatized to 1 per cent Ethyl Alcohol		Control
	Seconds	Seconds			Seconds	Seconds	
Exp. 1 cilia stop.....	430	240		Exp. 1 cilia stop.....	25	25	
2 cilia stop.....	240	360		2 cilia stop.....	50	35	
3 cilia stop.....	180	240		3 cilia stop.....	20	30	
4 cilia stop.....	270	105		4 cilia stop.....	45	30	
5 cilia stop.....	120	270		5 cilia stop.....	70	80	
6 cilia stop.....	210	360		6 cilia stop.....	35	40	
7 cilia stop.....	180	300		7 cilia stop.....	25	60	
8 cilia stop.....	360	270		8 cilia stop.....	30	30	
9 cilia stop.....	180	180		9 cilia stop.....	60	50	
10 cilia stop.....	180	270		10 cilia stop.....	45	65	
	—	—			—	—	
	240	259.5			40.5	44.5	

Thus remaining in ethyl alcohol did not increase the resistance to methyl alcohol. In both animals, indeed, the resistance to the methyl alcohol was slightly decreased by previous subjection to ethyl alcohol. The immunity due to the latter is in this case also specific.

While in glycerin and methyl alcohol there was often little evidence of decrease in resistance due to the previous acclimatization in ethyl alcohol, there was clearly no marked advantage or increase of resistance due to this cause. In both acid and alkali, on the other hand, the resistance of the animals was lowered in a marked degree by previous acclimatization to ethyl alcohol, although this had greatly increased the resistance to alcohol itself. This demonstrates in the clearest way the specificity of the immunity due to a residence in alcohol.

V SUMMARY AND CONCLUSIONS

The foregoing investigations consist of a study of the acclimatization of infusoria to ethyl alcohol, and the effects of this acclimatization on their resistance to other chemicals.

1 In certain strains of *Stentor coeruleus*, and in *Spirostomum ambiguum*, it was found that living for a few days in 1 per cent ethyl alcohol increases the resistance of the animals to a stronger solution of the same substance—ethyl alcohol. This increased resistance is shown (1) in the fact that the organisms are not so quickly killed in a lethal solution, (2) in the fact that they may continue to live in a solution stronger than that in which they could live before acclimatization.

2 Different species of infusoria and different strains of the same species, living under dissimilar environmental conditions showed various degrees of normal resistance to alcohol, and very different capacities for becoming acclimatized to it.

In *Stentor coeruleus* one strain designated as E manifested a high normal resistance, but this resistance was increased little or not at all by remaining in 1 per cent alcohol; while another strain F had a low normal resistance which was readily increased by living in a weak acclimatizing medium.

Incidentally, similar differences in the resistance of different sorts of infusoria to other chemicals were observed. Thus, *Spirostomum* withstood about eight times as concentrated a solution of hydrochloric acid as did *Stentor*. Marked differences are likewise observable among individuals of the same culture when tested for resistance to different chemicals.

3 Acclimatization to alcohol is shown not alone in an increase of resistance to a stronger solution but in changes in the behavior of the organisms. The unacclimatized animal responds to the stronger chemical by powerful motor reactions while the acclimatized organism shows much slighter activity.

4 In a weak solution of alcohol (1 per cent) the beginning of acclimatization is usually evident within a few hours. This increases in a fairly uniform ratio until about the fourth day, at which time a maximum degree of immunity may be expected.

5 The degree of resistance produced corresponds in a measure to the strength of the alcohol used as an acclimatizing medium. As compared with the resistance produced by a 1 per cent medium that due to $\frac{1}{2}$ per cent is lower in degree. In a medium much stronger than 1 per cent the correspondence does not hold, since stronger solutions decrease the resistance by producing injury to the organism.

6 The fact that in these experiments some strains show little or no capacity for becoming acclimatized to alcohol although tried for long periods of time and with refined methods makes it questionable whether acclimatization takes place so readily and to so high a degree as is commonly supposed.

Dr. Jennings informs me that extensive work in other chemicals carried on under his direction points to the same conclusion that I have just set forth—much of the work having given entirely negative results.

7 Tolerance, or acclimatization, to ethyl alcohol does not increase the resistance of the organisms to other chemicals. On the contrary it usually renders the animals *less* resistant to other agents. This matter was studied in detail in *Stentor* and *Spirostomum* for an acid, an alkali, and for two substances belonging to the group of alcohols, namely, glycerin and methyl alcohol. In all cases the animals which had acquired an increased resistance to ethyl alcohol as a result of living in a 1 per cent solution showed no increase or an actual decrease of resistance to other chemicals. Thus the immunity produced by ethyl alcohol is specific; it does not produce protection against all chemicals.

